PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/31, C07K 14/81, C11D 3/33, 3/386

A2

(11) International Publication Number: **WO 00/01826**

(43) International Publication Date: 13 January 2000 (13.01.00)

(21) International Application Number: PCT/US99/15246

(22) International Filing Date: 7 July 1999 (07.07.99)

(30) Priority Data:
60/091,911 7 July 1998 (07.07.98) US

(71) Applicant *(for all designated States except US)*: THE PROCTER & GAMBLE COMPANY [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SAUNDERS, Charles, Winston [US/US]; 5561 Carlsbad Court, Fairfield, OH 45014 (US). CORREA, Paul, Elliott [US/US]; 5755 Dry Ridge Road, Cincinnati, OH 45252 (US). SUN, Yiping [US/US]; 7589 Lakota Springs Drive, West Chester, OH 45069 (US). BAUER, Mark, Donald [US/US]; 6832 Richard Avenue, Cincinnati, OH 45224 (US). RUBINGH, Donn, Nelton [US/US]; 8113 Sheed Road, Cincinnati, OH 45247 (US).

(74) Agents: REED, T., David et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217–1087 (US).

(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: STABILIZED VARIANTS OF *STREPTOMYCES* SUBTILISIN INHIBITOR

(57) Abstract

The present invention relates to variants of *Streptomyces* subtilisin inhibitor (SSI) and those inhibitors having homology to SSI. Such variants are useful in conjunction with enzymes, particularly proteases, in cleaning compositions and personal care compositions. The variants comprise an amino acid substitution at position 63 corresponding to SSI. Such variants provide greater proteolytic stability in cleaning compositions and personal care compositions. The present invention also relates to cleaning compositions and personal care compositions comprising the present variants, as well as genes encoding the variants.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

STABILIZED VARIANTS OF *STREPTOMYCES* SUBTILISIN INHIBITOR

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/091,911, filed July 7, 1998.

FIELD OF THE INVENTION

The present invention relates to variants of *Streptomyces* subtilisin inhibitor (SSI) and those inhibitors having homology to SSI (SSI-like inhibitors). Such variants are useful in conjunction with enzymes, particularly proteases, in cleaning compositions and personal care compositions. The present invention also relates to cleaning compositions and personal care compositions comprising the present variants, as well as genes encoding the variants.

BACKGROUND OF THE INVENTION

Enzymes make up the largest class of naturally occurring proteins. One class of enzyme includes proteases which catalyze the hydrolysis of other proteins. This ability to hydrolyze proteins has been exploited by incorporating naturally occurring and protein engineered proteases into cleaning compositions, particularly those relevant to laundry applications. Furthermore, although explored to a lesser extent, others have incorporated such proteases into personal care compositions. During storage of the composition or even expression of the protease, however, the protease is frequently degraded by itself or may degrade other enzymes present in the composition. As a result of this degradation, the cleaning and personal care compositions have limited ability to achieve the intended enhanced performance.

It would therefore be beneficial to incorporate into the compositions an inhibitor of protease activity to limit protease autolysis and other degradation. It would be advantageous to provide reversible inhibitors of the protease, so that upon dilution of the composition during cleaning, or in the cleaning environment, the protease is no longer inhibited, but rather is available to hydrolyze proteinaceous stains. Furthermore, such inhibitors must be stable enough to adequately perform their inhibitory function.

Synthetic protease inhibitors or stabilizers have been disclosed for such uses, particularly in the laundry environment. For example, U.S. Patent No. 5,422,030, Panandiker et al., assigned to The Procter & Gamble Co., issued June 6, 1995, discloses aromatic borate esters to stabilize enzymes in laundry compositions. Furthermore, U.S. Patent No. 4,566,985, Bruno et al., assigned to Applied Biochemsists, Inc., issued January 28, 1986 proposes the use of benzamidine hydrochloride as an enzyme inhibitor. Such synthetic approaches to inhibition may provide longer shelf life, but may be expensive and may not improve isolation yield due to proteolysis in the fermentor.

Recognizing these shortcomings, those in the art have experimented with proteinaceous protease inhibitors to stabilize enzymes in cleaning compositions. Nature provides proteinaceous protease inhibitors to regulate the protease *in vivo*. However, because these naturally occurring proteinaceous protease inhibitors tend to be unstable, their commercial use in the presence of proteases and cleaning and personal care carriers may be somewhat limited.

Proteinaceous protease inhibitors are typically long peptides which bind to the active site of a protease and inhibit its activity. These inhibitors have typically been classified into several families (families I through IX) based on primary amino acid sequence homologies (See Laskowski et al., "Protein Inhibitors of Proteinases", Annual Review of Biochemistry, Vol. 49, pp. 593 - 626 (1980)). Included in these inhibitors are those commonly referred to as family VI inhibitors, including eglin and barley chymotrypsin inhibitor, and family III inhibitors, such as *Streptomyces* subtilisin inhibitor (SSI) and plasminostreptin.

Such inhibitors tend to bind to certain proteases better than others. Thus it is convenient to consider the inhibitor with a specific protease in mind. For this reason, the art often discusses "protease / peptide inhibitor pairs". An example of a known protease / peptide inhibitor pair is subtilisin BPN' / SSI. See e.g., Mitsui et al., "Crystal Structure of a Bacterial Protein Proteinase Inhibitor (*Streptomyces* Subtilisin Inhibitor) at 2.6 Å Resolution", Journal of Molecular Biology, Vol. 131, pp. 697 - 724 (1979) and Hirono et al., "Crystal Structure at 1.6 Å Resolution of the Complex of Subtilisin BPN' with

Streptomyces Subtilisin Inhibitor", Journal of Molecular Biology, Vol. 178, pp. 389 - 413 (1984).

SSI is stable in the presence of subtilisin BPN', as long as the inhibitor is present in sufficient amounts to inhibit all protease activity. However, it has been suggested that inhibitors having high affinity for protease do not dissociate upon dilution in the wash environment. See WO 92/03529, Mikkelson et al., assigned to Novo Nordisk A/S, published March 5, 1992.

However, if the binding constant (K_i) of an inhibitor provides for some protease activity in the cleaning composition containing the enzyme / inhibitor pair, the inhibitor, as well as enzymes in the composition, may be hydrolyzed. Therefore, it would be advantageous to find variants of SSI or other inhibitors which are suitably stable in the presence of protease as well as cleaning and personal care compositions. In addition, these inhibitors preferably have a preferred K_i for the particular protease to be inhibited. Such K_i should allow for inhibition of the protease in the final composition and during its storage. However, upon dilution of the cleaning or personal care composition or during the cleaning process, the protease and inhibitor should dissociate, allowing activity of the uninhibited protease.

Kojima et al., "Inhibition of Subtilisin BPN' by Reaction Site P1 Mutants of *Streptomyces* Subtilisin Inhibitor", Journal of Biochemistry, Vol. 109, pp. 377 - 382 (1991) made and measured the K_i of several SSI P1 position (position 73) variants using subtilisin BPN'. As another example, Mikkelsen et al. discloses mutations in family VI inhibitors that are said to lower binding affinity. WO 93/17086, Nielsen et al., assigned to Novo Nordisk A/S, published September 2, 1993, discloses mutations to plasminostreptin that are said to lower binding affinity.

However, stability of such protease inhibitors has been problematic. WO 98/13387, Correa et al., assigned to The Procter & Gamble Co., published April 2, 1998 (corresponding to U.S. Patent Application Serial No. 60/026,944) discloses variants which are disclosed as providing increased stability.

Despite the variety of approaches described in the art, there is a continuing need for more stable and effective protease inhibitors useful in cleaning and personal care

compositions. The present inventors have surprisingly discovered that SSI inhibitors, SSI-like inhibitors, and variants thereof are readily hydrolyzed between positions 63 and 64 corresponding to SSI during, for example, expression and / or in cleaning and personal care compositions. Accordingly, the present inventors herein provide variants of SSI inhibitors and SSI-like inhibitors which are modified, *inter alia*, at position 63 by a substituting amino acid residue. Such substitution imparts increased stability to the protease inhibitor. Such inhibitors are also advantageous because they bind protease at preferred levels as defined herein. The present invention therefore provides proteinaceous protease inhibitor variants having greater proteolytic stability, particularly in cleaning and personal care compositions, and lower affinity for the protease than the corresponding parent inhibitor.

SUMMARY OF THE INVENTION

The present invention provides variants having a modified amino acid sequence of a parent amino acid sequence, wherein the modified amino acid sequence comprises an amino acid substitution at position 63 corresponding to SSI, and wherein the parent amino acid sequence is selected from the group consisting of SSI, SSI-like inhibitors, variants of SSI, and variants of SSI-like inhibitors. Such variants are useful, for example, for inhibiting proteases, particularly during storage or expression. The present invention also relates to genes encoding such variants and cleaning and personal care compositions comprising such variants.

DETAILED DESCRIPTION OF THE INVENTION

The essential components of the present invention are described herein. Also included are non-limiting descriptions of various optional and preferred components useful in the embodiments of the present invention.

The present invention can comprise, consist of, or consist essentially of, any of the required or optional components, ingredients, and / or limitations described herein.

All percentages and ratios are calculated by weight unless otherwise indicated. All percentages are calculated based on the total composition unless otherwise indicated.

Referred to herein are trade names for materials including, but not limited to, proteases and optional components. The inventors herein do not intend to be limited by

materials under a certain trade name. Equivalent materials (*e.g.*, those obtained from a different source under a different name or catalog (reference) number) to those referenced by trade name may be substituted and utilized in the compositions herein.

All component, ingredient, or composition levels are in reference to the active level of that component, ingredient, or composition, and are exclusive of impurities, for example, residual solvents or by-products, which may be present in commercially available sources.

All documents referred to herein, including all patents, patent applications, and printed publications, are hereby incorporated by reference in their entirety.

As used herein, abbreviations will be used to describe amino acids. Table I provides a list of abbreviations used herein:

Table I

Amino Acid	Three-letter Abbreviation	One-letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Definitions

As used herein, the term "mutation" refers to alterations in gene sequences and amino acid sequences produced by those gene sequences. Mutations may be deletions, substitutions, or additions of amino acid residues to the wild-type or parent sequence.

As used herein, the term "parent" refers to a protease, protease inhibitor, protein, or peptide, wild-type or variant, with no amino acid substitution at position 63 corresponding to SSI (*i.e.*, the amino acid substitution at position 63 is naturally occurring). An example of one of these parents is an inhibitor known as *Streptomyces* Subtilisin Inhibitor (SSI) (represented by SEQ ID NO: 1). SSI is further described by Ikenaka et al., "Amino Acid Sequence of an Alkaline Proteinase Inhibitor (*Streptomyces* Subtilisin Inhibitor) from *Streptomyces albogriseoulus* S-3253", Journal of Biochemistry, Vol. 76, pp. 1191 - 1209 (1974). As used herein, the amino acid numbering of SSI is that of Ikenaka et al. The present inventors also use a synthetic SSI gene, designed to be rich in adenine and thymine, as is *B. subtilis* DNA. This synthetic

gene encodes four extra amino acid residues at the amino terminus of the peptide due to expression plasmid construction methods. This modified amino acid sequence, including these four additional amino acids, is represented by SEQ ID NO: 2.

As used herein, the term "wild-type" refers to a protein or peptide, herein specifically a protease or protease inhibitor, produced by unmutated organisms.

As used herein, the term "variant" means a protein or peptide, herein specifically a protease inhibitor or protease, having an amino acid sequence which differs from that of the parent protease inhibitor or protease, respectively.

Variants of the Present Invention

The present inventors have discovered variants of protease inhibitors which are more stable, for example, *in vitro* and in the presence of cleaning and personal care composition materials. In addition, such variants may be more stable *in vivo* as well, thus increasing the yield of protease from the organism.

The present inventors have also discovered variants exhibiting preferred binding constants (K_i), which provide for suitable inhibition of protease during growth, harvesting, purification, storage, and during the cleaning process. Such preferred binding provides for better stability and longer shelf life.

The variants of the present invention have improved stability to proteases, and inhibit protease in a cleaning or personal care composition, but dissociate upon dilution in the cleaning environment.

The present variants have a modified amino acid sequence of a parent amino acid sequence, wherein the modified amino acid sequence comprises an amino acid substitution at position 63 corresponding to *Streptomyces* subtilisin inhibitor (herein referred to as SSI), and wherein the parent amino acid sequence is selected from SSI, SSI-like inhibitors, variants of SSI, and variants of SSI-like inhibitors. The substitution at position 63 corresponding to SSI may be with any amino acid residue which imparts increased stability relative to the parent amino acid sequence. Most preferably, the substitution at position 63 corresponding to SSI is with isoleucine. Such a variant may be represented as "L63I". In describing this variant, the original amino acid occurring in the parent amino acid sequence is given first, the position number second, and the substituted amino acid third. Thus, L63I means that the leucine (L) which appeared as the sixty-third amino acid position (position 63) in the native inhibitor SSI is replaced with isoleucine (I). The position numbering corresponds to that of Ikenaka et al., *supra* (SEQ ID NO: 1), and ignores the four additional amino acid residues present at the amino terminus of the synthetic SSI (SEQ ID NO: 2). Such representations for other substitutions listed herein are presented in a consistent manner.

The variants herein are not limited to SSI substituted at position 63. Rather, the substitution at position 63 may also be made in parent amino acid sequences (including,

of course, the nucleotide sequences coding for that amino acid sequence) wherein the parent is itself a variant of SSI, an SSI-like inhibitor, or a variant of SSI-like inhibitors. The more preferred parent amino acid sequences include SSI and variants of SSI. The most preferred parent amino acid sequences are variants of SSI. Variants of SSI have been disclosed in, for example, Kojima et al., "Inhibition of Subtilisin BPN' by Reaction Site P1 Mutants of *Streptomyces* Subtilisin Inhibitor", Journal of Biochemistry, Vol. 109, pp. 377 - 382 (1991); Tamura et al., "Mechanisms of Temporary Inhibition in *Streptomyces* Subtilisin Inhibitor Induced by an Amino Acid Substitution, Tryptophan 86 Replaced by Histidine", Biochemistry, Vol. 30, pp. 5275 - 5286 (1991); JO 3099-099-A, assigned to Tsumura & Co., published September 12, 1989; Mikkelsen et al., U.S. Patent No. 5,674,833, assigned to Novo Nordisk A/S, issued October 7, 1997; and WO 93/17086, Nielsen et al., assigned to Novo Nordisk A/S, published September 2, 1993. Other variants of SSI have been disclosed in U.S. Patent Application Serial No. 60/026,944, Correa et al., corresponding to WO 98/13387, Correa et al., assigned to The Procter & Gamble Co., published April 2, 1998, such variants herein being collectively described as "Inhibitor Group A". Preferred variants of SSI (for use as parent amino acid sequences herein) are those of Inhibitor Group A. More preferred variants which are useful as the parent amino acid sequences herein are listed in the following Tables 2 - 6. Again, all position numbering corresponds to SSI as described by Ikenaka et al.

Table 2

Non-limiting Examples of Parent Amino Acid Sequences Having a Single Substitution

Parent 1	D83C
Parent 4	M73D
Parent 34	M73P

Table 3

Non-limiting Examples of Parent Amino Acid Sequences Having Double Substitutions

Parent 2	M73D + D83C
Parent 3	M73P + D83C
Parent 5	M70Q + D83C
Parent 29	M73P + S98D
Parent 30	M73P + S98E
Parent 31	M73P + S98A

Table 4

Non-limiting Examples of Parent Amino Acid Sequences Having Triple Substitutions

Parent 6	M73P + D83C + S98A
Parent 7	M73P + Y75A + D83C
Parent 8	M73P + D83C + S98V
Parent 9	M70Q + M73P + D83C
Parent 10	M73P + V74A + D83C
Parent 11	M73P + V74F + D83C
Parent 12	M70Q + D83C + S98A
Parent 13	G47D + M70Q + D83C
Parent 14	G47D + D83C + S98A
Parent 15	G47D + M73P + D83C
Parent 16	G47D + M73D + D83C
Parent 27	M73P + D83C + S98D
Parent 28	M73P + D83C + S98E

Table 5

Non-limiting Examples of Parent Amino Acid Sequences Having Quadruple Substitutions

Parent 17	M70Q + M73P + V74F + D83C
Parent 18	M70Q + M73P + V74W + D83C
Parent 19	M70Q + M73P + D83C + S98A
Parent 20	G47D + M73P + V74F + D83C
Parent 21	G47D + M73P + V74W + D83C
Parent 22	G47D + M73P + D83C + S98A
Parent 32	G47D + M73P + D83C + S98D
Parent 33	G47D + M73P + D83C + S98E

Table 6

Non-limiting Examples of Parent Amino Acid Sequences Having Quintuple Substitutions

Parent 23	G47D + M70Q + M73P + V74F + D83C
Parent 24	G47D + M70Q + M73P + V74W + D83C
Parent 25	G47D + M73P + V74F + D83C + S98A
Parent 26	G47D + M73P + V74W + D83C + S98A

Thus, non-limiting examples of variants of the present invention may be described as Variant 1, Variant 2, *etc.*, wherein, for example, Variant 1 may be

represented as L63* + D83C, wherein "*" represents any amino acid other than that originally occurring at the position corresponding to 63 in SSI, and wherein Variant 1 - I may be represented as L63I + D83C. Accordingly, preferred variants of the present invention are listed in the following Table 7. Even more preferred among those variants listed in Table 7 are those having isoleucine substituting at position 63.

Table 7

Non-limiting Examples of Preferred Variants of the Present Invention

Variant 1	L63* + D83C
Variant 4	L63* + M73D
Variant 1 - I	L63I + D83C
Variant 4 - I	L63I + M73D
Variant 2	L63* + M73D + D83C
Variant 3	L63* + M73P + D83C
Variant 5	L63* + M70Q + D83C
Variant 2 - I	L63I + M73D + D83C
Variant 3 - I	L63I + M73P + D83C
Variant 5 - I	L63I + M70Q + D83C
Variant 6	L63* + M73P + D83C + S98A
Variant 7	L63* + M73P + Y75A + D83C
Variant 8	L63* + M73P + D83C + S98V
Variant 9	L63* + M70Q + M73P + D83C
Variant 10	L63* + M73P + V74A + D83C
Variant 11	L63* + M73P + V74F + D83C
Variant 12	L63* + M70Q + D83C + S98A
Variant 13	L63* + G47D + M70Q + D83C
Variant 14	L63* + G47D + D83C + S98A
Variant 15	L63* + G47D + M73P + D83C
Variant 16	L63* + G47D + M73D + D83C
Variant 6 - I	L63I + M73P + D83C + S98A
Variant 7 - I	L63I + M73P + Y75A + D83C
Variant 8 - I	L63I + M73P + D83C + S98V
Variant 9 - I	L63I + M70Q + M73P + D83C
Variant 10 - I	L63I + M73P + V74A + D83C
Variant 11 - I	L63I + M73P + V74F + D83C
Variant 12 - I	L63I + M70Q + D83C + S98A
Variant 13 - I	L63I + G47D + M70Q + D83C
Variant 14 - I	L63I + G47D + D83C + S98A
Variant 15 - I	L63I + G47D + M73P + D83C
Variant 16 - I	L63I + G47D + M73D + D83C
Variant 17	L63* + M70Q + M73P + V74F + D83C

Variant 18	L63* + M70Q + M73P + V74W + D83C
Variant 19	L63* + M70Q + M73P + D83C + S98A
Variant 20	L63* + G47D + M73P + V74F + D83C
Variant 21	L63* + G47D + M73P + V74W + D83C
Variant 22	L63* + G47D + M73P + D83C + S98A
Variant 17 - I	L63I + M70Q + M73P + V74F + D83C
Variant 18 - I	L63I + M70Q + M73P + V74W + D83C
Variant 19 - I	L63I + M70Q + M73P + D83C + S98A
Variant 20 - I	L63I + G47D + M73P + V74F + D83C
Variant 21 - I	L63I + G47D + M73P + V74W + D83C
Variant 22 - I	L63I + G47D + M73P + D83C + S98A
Variant 23	L63* + G47D + M70Q + M73P + V74F + D83C
Variant 24	L63* + G47D + M70Q + M73P + V74W + D83C
Variant 25	L63* + G47D + M73P + V74F + D83C + S98A
Variant 26	L63* + G47D + M73P + V74W + D83C + S98A
Variant 23 - I	L63I + G47D + M70Q + M73P + V74F + D83C
Variant 24 - I	L63I + G47D + M70Q + M73P + V74W + D83C
Variant 25 - I	L63I + G47D + M73P + V74F + D83C + S98A
Variant 26 - I	L63I + G47D + M73P + V74W + D83C + S98A
Variant 27 - I	L63I + M73P + D83C + S98D
Variant 28 - I	L63I + M73P + D83C + S98E
Variant 29 - I	L63I + M73P + S98D
Variant 30 - I	L63I + M73P + S98E
Variant 31 - I	L63I + M73P + S98A
Variant 32 - I	L63I + G47D + M73P + D83C + S98D
Variant 33 - I	L63I + G47D + M73P + D83C + S98E
Variant 34 - I	L63I + M73P

Other preferred parent amino acid sequences herein include those comprising a substitution at position 62 corresponding to SSI. The substitution at position 62 may be any amino acid residue other than that occurring naturally in the parent (in the case of SSI, the naturally occurring amino acid residue is alanine). Preferably, the substituting amino acid at position 62 is selected from Lys, Arg, Glu, Asp, Thr, Ser, Gln, Asn, and Trp, more preferably Lys, Arg, Glu, Asp, Thr, Ser, Gln, and Asn, still more preferably Lys, Arg, Glu, and Asp, even more preferably Lys and Arg, and most preferably Lys. Preferred parent amino acid sequences herein have a substitution at position 62 in addition to the substitutions listed in Tables 2 - 6. Examples of such parents are designated as Parent X - A62*, wherein the "X" corresponds to the parent exemplified in Tables 2 - 6. Thus, Parent 6 - A62* corresponds to A62* + M73P + D83C + S98A.

Similarly, Parent 6 - A62K corresponds to A62K + M73P + D83C + S98A. Similarly, an exemplified variant of the present invention is Variant 6 - I - A62*, which corresponds to to A62* + L63I + M73P + D83C + S98A. Thus, Variant 6 - I - A62K corresponds to A62K + L63I + M73P + D83C + S98A. In this fashion, Table 8 lists other preferred variants of the present invention.

Table 8

Non-limiting Examples of Preferred Variants of the Present Invention

Variant 1 - A62*	A62* + L63* + D83C
Variant 4 - A62*	A62* + L63* + M73D
Variant 1 - I - A62*	A62* + L63I + D83C
Variant 4 - I - A62*	A62* + L63I + M73D
Variant 4 - I - A62K	A62K + L63I + M73D
Variant 4 - I - A62R	A62R + L63I + M73D
Variant 2 - A62*	A62* + L63* + M73D + D83C
Variant 3 - A62*	A62* + L63* + M73P + D83C
Variant 5 - A62*	A62* + L63* + M70Q + D83C
Variant 2 - I - A62*	A62* + L63I + M73D + D83C
Variant 3 - I - A62*	A62* + L63I + M73P + D83C
Variant 5 - I - A62*	A62* + L63I + M70Q + D83C
Variant 2 - I - A62K	A62K + L63I + M73D + D83C
Variant 2 - I - A62R	A62R + L63I + M73D + D83C
Variant 3 - I - A62K	A62K + L63I + M73P + D83C
Variant 3 - I - A62R	A62R + L63I + M73P + D83C
Variant 5 - I - A62K	A62K + L63I + M70Q + D83C
Variant 5 - I - A62R	A62R + L63I + M70Q + D83C
Variant 6 - A62*	A62* + L63* + M73P + D83C + S98A
Variant 7 - A62*	A62* + L63* + M73P + Y75A + D83C
Variant 8 - A62*	A62* + L63* + M73P + D83C + S98V
Variant 9 - A62*	A62* + L63* + M70Q + M73P + D83C
Variant 10 - A62*	A62* + L63* + M73P + V74A + D83C
Variant 11 - A62*	A62* + L63* + M73P + V74F + D83C
Variant 12 - A62*	A62* + L63* + M70Q + D83C + S98A
Variant 13 - A62*	A62* + L63* + G47D + M70Q + D83C
Variant 14 - A62*	A62* + L63* + G47D + D83C + S98A
Variant 15 - A62*	A62* + L63* + G47D + M73P + D83C
Variant 16 - A62*	A62* + L63* + G47D + M73D + D83C
Variant 6 - I - A62*	A62* + L63I + M73P + D83C + S98A
Variant 6 - I - A62K	A62K + L63I + M73P + D83C + S98A
Variant 6 - I - A62R	A62R + L63I + M73P + D83C + S98A
Variant 7 - I - A62*	A62* + L63I + M73P + Y75A + D83C

Variant 7 - I - A62K	A62K + L63I + M73P + Y75A + D83C
Variant 7 - I - A62R	A62R + L63I + M73P + Y75A + D83C
Variant 8 - I - A62*	A62* + L63I + M73P + D83C + S98V
Variant 8 - I - A62K	A62K + L63I + M73P + D83C + S98V
Variant 8 - I - A62R	A62R + L63I + M73P + D83C + S98V
Variant 9 - I - A62*	A62* + L63I + M70Q + M73P + D83C
Variant 9 - I - A62K	A62K + L63I + M70Q + M73P + D83C
Variant 9 - I - A62R	A62R + L63I + M70Q + M73P + D83C
Variant 10 - I - A62*	A62* + L63I + M73P + V74A + D83C
Variant 10 - I - A62K	A62K + L63I + M73P + V74A + D83C
Variant 10 - I - A62R	A62R + L63I + M73P + V74A + D83C
Variant 11 - I - A62*	A62* + L63I + M73P + V74F + D83C
Variant 11 - I - A62K	A62K + L63I + M73P + V74F + D83C
Variant 11 - I - A62R	A62R + L63I + M73P + V74F + D83C
Variant 12 - I - A62*	A62* + L63I + M70Q + D83C + S98A
Variant 12 - I - A62K	A62K + L63I + M70Q + D83C + S98A
Variant 12 - I - A62R	A62R + L63I + M70Q + D83C + S98A
Variant 13 - I - A62*	A62* + L63I + G47D + M70Q + D83C
Variant 13 - I - A62K	A62K + L63I + G47D + M70Q + D83C
Variant 13 - I - A62R	A62R + L63I + G47D + M70Q + D83C
Variant 14 - I - A62*	A62* + L63I + G47D + D83C + S98A
Variant 14 - I - A62K	A62K + L63I + G47D + D83C + S98A
Variant 14 - I - A62R	A62R + L63I + G47D + D83C + S98A
Variant 15 - I - A62*	A62* + L63I + G47D + M73P + D83C
Variant 15 - I - A62K	A62K + L63I + G47D + M73P + D83C
Variant 15 - I - A62R	A62R + L63I + G47D + M73P + D83C
Variant 16 - I - A62*	A62* + L63I + G47D + M73D + D83C
Variant 16 - I - A62K	A62K + L63I + G47D + M73D + D83C
Variant 16 - I - A62R	A62R + L63I + G47D + M73D + D83C
Variant 17 - A62*	A62* + L63* + M70Q + M73P + V74F + D83C
Variant 18 - A62*	A62* + L63* + M70Q + M73P + V74W + D83C
Variant 19 - A62*	A62* + L63* + M70Q + M73P + D83C + S98A
Variant 20 - A62*	A62* + L63* + G47D + M73P + V74F + D83C
Variant 21 - A62*	A62* + L63* + G47D + M73P + V74W + D83C
Variant 22 - A62*	A62* + L63* + G47D + M73P + D83C + S98A
Variant 17 - I - A62*	A62* + L63I + M70Q + M73P + V74F + D83C
Variant 17 - I - A62K	A62K + L63I + M70Q + M73P + V74F + D83C
Variant 17 - I - A62R	A62R + L63I + M70Q + M73P + V74F + D83C
Variant 18 - I - A62*	A62* + L63I + M70Q + M73P + V74W + D83C
Variant 18 - I - A62K	A62K + L63I + M70Q + M73P + V74W + D83C
Variant 18 - I - A62R	A62R + L63I + M70Q + M73P + V74W + D83C
Variant 19 - I - A62*	A62* + L63I + M70Q + M73P + D83C + S98A
Variant 19 - I - A62K	A62K + L63I + M70Q + M73P + D83C + S98A

Variant 19 - I - A62R	A62R + L63I + M70Q + M73P + D83C + S98A
Variant 20 - I - A62*	A62* + L63I + G47D + M73P + V74F + D83C
Variant 20 - I - A62K	A62K + L63I + G47D + M73P + V74F + D83C
Variant 20 - I - A62R	A62R + L63I + G47D + M73P + V74F + D83C
Variant 21 - I - A62*	A62* + L63I + G47D + M73P + V74W + D83C
Variant 21 - I - A62K	A62K + L63I + G47D + M73P + V74W + D83C
Variant 21 - I - A62R	A62R + L63I + G47D + M73P + V74W + D83C
Variant 22 - I - A62*	A62* + L63I + G47D + M73P + D83C + S98A
Variant 22 - I - A62K	A62K + L63I + G47D + M73P + D83C + S98A
Variant 22 - I - A62R	A62R + L63I + G47D + M73P + D83C + S98A
Variant 23 - A62*	A62* + L63* + G47D + M70Q + M73P + V74F + D83C
Variant 24 - A62*	A62* + L63* + G47D + M70Q + M73P + V74W + D83C
Variant 25 - A62*	A62* + L63* + G47D + M73P + V74F + D83C + S98A
Variant 26 - A62*	A62* + L63* + G47D + M73P + V74W + D83C + S98A
Variant 23 - I - A62*	A62* + L63I + G47D + M70Q + M73P + V74F + D83C
Variant 23 - I - A62K	A62K + L63I + G47D + M70Q + M73P + V74F + D83C
Variant 23 - I - A62R	A62R + L63I + G47D + M70Q + M73P + V74F + D83C
Variant 24 - I - A62*	A62* + L63I + G47D + M70Q + M73P + V74W + D83C
Variant 24 - I - A62K	A62K + L63I + G47D + M70Q + M73P + V74W + D83C
Variant 24 - I - A62R	A62R + L63I + G47D + M70Q + M73P + V74W + D83C
Variant 25 - I - A62*	A62* + L63I + G47D + M73P + V74F + D83C + S98A
Variant 25 - I - A62K	A62K + L63I + G47D + M73P + V74F + D83C + S98A
Variant 25 - I - A62R	A62R + L63I + G47D + M73P + V74F + D83C + S98A
Variant 26 - I - A62*	A62* + L63I + G47D + M73P + V74W + D83C + S98A
Variant 26 - I - A62K	A62K + L63I + G47D + M73P + V74W + D83C + S98A
Variant 26 - I - A62R	A62R + L63I + G47D + M73P + V74W + D83C + S98A
Variant 27 - I - A62K	A62K + L63I + M73P + D83C + S98D
Variant 27 - I - A62R	A62R + L63I + M73P + D83C + S98D
Variant 28 - I - A62K	A62K + L63I + M73P + D83C + S98E
Variant 28 - I - A62R	A62R + L63I + M73P + D83C + S98E
Variant 29 - I - A62K	A62K + L63I + M73P + S98A
Variant 29 - I - A62R	A62R + L63I + M73P + S98A
Variant 30 - I - A62K	A62K + L63I + M73P + S98D
Variant 30 - I - A62R	A62R + L63I + M73P + S98D
Variant 31 - I - A62K	A62K + L63I + M73P + S98E
Variant 31 - I - A62R	A62R + L63I + M73P + S98E

Other preferred parent amino acid sequences (which are variants of SSI) useful in the present invention include those having a single substitution at position 98 corresponding to SSI and those having a double substitution, one at position 62 and one at position 98. Table 9 lists preferred parent amino acid sequences in this class.

Table 9

Non-limiting Examples of Parent Amino Acid Sequences

Parent 32	A62K + S98Q
Parent 33	A62K + S98D
Parent 34	A62K + S98E
Parent 35	A62R + S98Q
Parent 36	A62R + S98D
Parent 37	A62R + S98E
Parent 38	S98A
Parent 39	A62K + S98A
Parent 40	A62R + S98A
Parent 41	S98Q
Parent 42	S98D
Parent 43	S98E

The corresponding examples of variants of the present invention are listed in the following Table 10.

Table 10

Non-limiting Examples of Variants of the Present Invention

Variant 32	L63I + A62K + S98Q
Variant 33	L63I + A62K + S98D
Variant 34	L63I + A62K + S98E
Variant 35	L63I + A62R + S98Q
Variant 36	L63I + A62R + S98D
Variant 37	L63I + A62R + S98E
Variant 38	L63I + S98A
Variant 39	A62K + L63I + S98A
Variant 40	A62R + L63I + S98A
Variant 41	L63I + S98Q
Variant 42	L63I + S98D
Variant 43	L63I + S98E

SSI may exist in dimeric form. Thus without being bound by theory, it is possible that stabilizing dimeric SSI provides increased protease resistance to excess protease. Preferably this stabilized dimeric SSI variant is composed of two SSI variant monomers covalently bound together. This may be by ester, amido, disulfide, or other linkages, commonly occurring in amino acids and their sidechains. Thus "covalent

dimerization" and "covalent stabilization" refers to such covalently bound monomers, which form the dimer. Preferably this dimerization occurs *via* disulfide bonds. The variants of the present invention are meant to include those existing in dimeric form, whether by intramolecular or intermolecular forces.

Other parent amino acid sequences which are useful herein include SSI-like inhibitors (often referred to as SSI-like (SIL) proteins) and variants of SSI-like inhibitors. Background information relating to SSI-like inhibitors may be found in Laskowski et al., "Protein Inhibitors of Proteases", Annual Review of Biochemistry, Vol. 49, pp. 593 - 626 (1980). Preferred SSI-like inhibitors have greater than about 50%, preferably greater than about 65%, and more preferably greater than about 70% amino acid sequence identity with SSI, preferably wherein the inhibitor may be classified as a family III inhibitor. See Laskowski et al., *supra.* Examples of such SSI-like inhibitors include SIL10 (the sequence of which is provided as SEQ ID NO: 4) , SIL13 (SEQ ID NO: 5), and SIL14 (SEQ ID NO: 6), each of which are further described in Terabe et al., "Three Novel Subtilisin-Trypsin Inhibitors from *Streptomyces*: Primary Structures and Inhibitory Properties", Journal of Biochemistry, Vol. 116, pp. 1156 - 1163 (1994), and SIL2 (the sequence of which is provided as SEQ ID NO: 9), SIL3 (SEQ ID NO: 10), and SIL4 (SEQ ID NO: 11), each of which are further described by Taguchi et al., "Comparative Studies on the Primary Structures and Inhibitory Properties of Subtilisin-trypsin Inhibitors from *Streptomyces*", European Journal of Biochemistry, Vol. 220, pp. 911 - 918 (1994). Two other examples of such SSI-like inhibitors include STI1 (the sequence of which is provided as SEQ ID NO: 7) and STI2 (SEQ ID NO: 8), which are further described in Strickler et al., "Two Novel *Streptomyces* Protein Protease Inhibitors", The Journal of Biological Chemistry, Vol. 267, No. 5, pp. 3236 - 3241 (1992). Another SSI-like inhibitor is known as plasminostreptin (the sequence of which is provided as SEQ ID NO: 12) which is further described in Sugino et al., "Plasminostreptin, a Protein Proteinase Inhibitor Produced by *Streptomyces antifibrinolyticus*", The Journal of Biological Chemistry, Vol. 253, No. 5, pp. 1546 - 1555 (1978). Still another SSI-like inhibitor is SLPI (the sequence of which is provided as SEQ ID NO: 13) which is further described in Ueda et al., "A Protease Inhibitor Produced by *Streptomyces lividans* 66 Exhibits Inhibitory Activities Toward Both

Subtilisin BPN' and Trypsin", Journal of Biochemistry, Vol. 112, pp. 204 - 211 (1993). Still another SSI-like inhibitor is SAC I (the sequence of which is provided as SEQ ID NO: 14) which is further described in Tanabe et al., "Primary Structure and Reactive Site of *Streptoverticillium* Anticoagulant (SAC), a Novel Protein Inhibitor of Blood Coagulation Produced by *Streptoverticillium cinnamoneum* subsp. *cinnamoneum*", Journal of Biochemistry, Vol. 115, pp. 752 - 761 (1994). Still another SSI-like inhibitor is SIL1 (the sequence of which is provided as SEQ ID NO: 15) which is further described in Kojima et al., "Primary Structure and Inhibitory Properties of a Proteinase Inhibitor Produced by *Streptomyces cacaoi*", Biochimica et Biophysica Acta, Vol. 1207, pp. 120 - 125 (1994). Other SSI-like inhibitors are discussed in Taguchi et al., "High Frequency of SSI-Like Protease Inhibitors Among *Streptomyces*", Bioscience, Biotechnology, and Biochemistry, Vol. 57, pp. 522 - 524 (1993), Taguchi et al., "*Streptomyces* Subtilisin Inhibitor-Like Proteins Are Distributed Widely in Streptomycetes", Applied and Environmental Microbiology, pp. 4338 - 4341 (Dec. 1993), and Suzuki et al., "Partial Amino Acid Sequence of an Alkaline Protease Inhibitor", Agricultural Biological Chemistry, Vol. 45, pp. 629 - 634 (1981). As one skilled in the art will understand, still other SSI-like inhibitors are described in the art.

Variants of SSI-like inhibitors may also be utilized as parent amino acid sequences herein. Such variants include those having one or more mutations in the amino acid sequence of a selected SSI-like inhibitor as described herein, *supra*. Among others, all of the substitutions exemplified in the variants shown herein may also be made at corresponding positions in SSI-like inhibitors to provide a parent amino acid sequence. Other non-limiting examples of variants of SSI-like inhibitors which may be utilized as parent amino acid sequences are disclosed in Nielsen et al., WO 93/17086, assigned to Novo Nordisk A/S, published September 2, 1993.

As one skilled in the art will understand, position 63 (for example) of an SSI-like inhibitor, variant thereof, or variant of SSI, using its native numbering, may not correspond to position 63 of SSI. Accordingly, as is understood readily in the art, sequence numbering may need adjustment to locate the position which corresponds to that of position 63 (for example) of SSI. Sequence alignments are readily found in the references cited herein as well as other references in the art.

Preferably, the present variants exhibit a K_i which allows the variant to inhibit nearly all protease (preferably greater than about 60%, more preferably about 99%) in the cleaning or personal care compositions, but dissociate from the protease upon dilution and / or during the cleaning process.

For example, wherein a 2:1 stoichiometry of variant to protease is used, it is preferred that inhibitors exhibit a K_i against the protease from about 10^{-12} M to about 10^{-4} M, more preferably from about 10^{-10} M to about 10^{-6} M, and most preferably from about 10^{-8} M to about 10^{-7} M. Of course, should washing machine dimensions or product concentrations change, the K_i is adjusted accordingly. Prediction of a useful K_i range is readily determined by the skilled artisan without undue experimentation by considering such parameters as dilution of the composition upon use, temperature dependence of the binding constant in relation to the temperature of cleaning method used, stoichiometry of the inhibitor to the protease, and the like.

Since the variant is ultimately encoded *in vivo* by DNA, the DNA can be used to define the sequence of the variant. The DNA, which codes for the variant, can be used in any number of plasmids and / or expression systems, including *in vitro* expression systems and *in vivo* systems such as plants, (preferably those used in biotechnology, including tobacco, oilseed plants, such as rapeseed, soybean and the like, grain, such as maize, barley, oats, other vegetables, such as tomatoes, potatoes and the like) and microorganisms, including fungi, such as yeast, and bacteria, such as *Bacillus, E. coli*, and the like. Preferably the expression system is a microorganism, more preferably bacterial in nature, most preferably *E. coli* or *Bacillus*, still more preferably *Bacillus*.

The DNA encoding the variant may be incorporated into a plasmid or phage, active in the cell, or may be incorporated directly into the genome of the organism which is used in cloning or expression of the variants of the invention.

It should be understood that the skilled artisan, given the instruction of this invention, will appreciate that the DNA used to code for a present variant may be placed in the same plasmid, phage, or chromosome as other variants of the invention. In addition, such plasmids, phages or chromosomes may also encode proteases, including

fusion proteins which include as part of the fusion protein an inhibitor and / or protease, which may or may not be inhibited by the variant of the invention.

It is also well understood by the skilled artisan that the DNA described above also contemplates, and discloses the RNA transcript of the DNA. The skilled artisan can of course, without experimentation, know the RNA sequence, by inspection of the DNA sequence.

The present invention also relates to genes encoding the present variants.

It is also contemplated that the skilled artisan may desire to prepare antibodies to the variants of the present invention. These antibodies may be prepared using known methodologies.

For example, the variants of the present invention can be injected into suitable mammalian subjects such as mice, rabbits, and the like. Suitable protocols involve repeated injection of the immunogen in the presence of adjuvants according to a schedule which boosts production of antibodies in the serum. The titers of the immune serum can readily be measured using immunoassay procedures, now standard in the art, employing the invention variants as antigens.

The antisera obtained may be used directly or monoclonal antibodies may be obtained by harvesting the peripheral blood lymphocytes or the spleen of the immunized animal and immortalizing the antibody-producing cells, followed by identifying the suitable antibody producers using standard immunoassay techniques.

The polyclonal or monoclonal preparations are then useful in monitoring expression of the invention, using standard test methodologies. Thus it is also envisioned that a kit may be prepared using these antibodies for one to use to determine expression levels and the like.

Such antibodies can also be coupled to labels such as scintigraphic labels, *e.g.*, technetium 99 or I-131, or fluorescent labels, using standard coupling methods. The labeled antibodies can also be used in competitive assays, such as kinetic assays used to determine K_i.

As is recognized in the art, there are occasionally errors in DNA and amino acid sequencing methods. As a result, one of ordinary skill in the art reproducing the present

inventors' work from the disclosure herein can discover any sequencing errors using routine skill, and make changes as appropriate.

Method of Making and Using

The following examples are not meant to limit the claimed invention in any way, but rather provide the skilled artisan with guidance as to how to make and use the invention. Given the guidance of the examples, the other disclosure herein, and the information readily available to those skilled in the art, the skilled artisan is able to make and use the invention. For brevity, exhaustive recitation of the art and art known methodologies and the like are eliminated, as these are well within the purview of the skilled artisan.

The variants may be prepared by mutating the nucleotide sequences that code for a parent amino acid sequence, thereby resulting in variants having modified amino acid sequences. Such methods are well-known in the art; one such method is as follows.

A phagemid containing the gene corresponding to the parent amino acid sequence is used to transform *Escherichia coli dut- ung-* strain CJ236 and a single stranded uracil-containing DNA template is produced using the VCSM13 helper phage (Kunkel et al., "Rapid and Efficient Site-Specific Mutagenesis Without Phenotypic Selection", Methods in Enzymology, Vol 154, pp. 367 - 382 (1987), as modified by Yuckenberg et al., "Site-Directed *in vitro* Mutagenesis Using Uracil-Containing DNA and Phagemid Vectors", Directed Mutagenesis - A Practical Approach, McPherson, M. J. ed., pp. 27 - 48 (1991). Primer site-directed mutagenesis modified from the method of Zoller and Smith (Zoller, M. J., and M. Smith, "Oligonucleotide - Directed Mutagenesis Using M13 - Derived Vectors: An Efficient and General Procedure for the Production of Point Mutations in any Fragment of DNA", Nucleic Acids Research, Vol. 10, pp. 6487 - 6500 (1982) is used to produce all variants (essentially as presented by Yuckenberg et al., *supra*).

Oligonucleotides are made using a 380B DNA synthesizer (Applied Biosystems Inc.). Mutagenesis reaction products are transformed into *Escherichia coli* strain MM294 (American Type Culture Collection *E. coli* 33625). All mutations are confirmed by DNA sequencing and the isolated DNA is transformed into the *Bacillus subtilis* expression strain PG632 (Saunders et al., "Optimization of the Signal-Sequence Cleavage Site for Secretion from *Bacillus subtilis* of a 34-amino acid Fragment of

Human Parathyroid Hormone", Gene, Vol. 102, pp. 277 - 282 (1991) and Yang et al., "Cloning of the Neutral Protease Gene of *Bacillus subtilis* and the Use of the Cloned Gene to Create an *in vitro* - Derived Deletion Mutation", Journal of Bacteriology, Vol. 160, pp. 15 - 21 (1984).

Variant preparations are made as follows. *Bacillus subtilis* cells containing the plasmid of interest are cultured in medium with 20 g/l tryptone, 20 g/l yeast extract, and 5 g/l of sodium chloride supplemented with 1.25% maltrin M100 (Grain Processing Corporation, Muscatine, IA), 100 mM HEPES pH 7.5, 80 μM $MnCl_2$, and 50 μM kanamycin. The cultures are incubated for 24 hours at 37°C.

The variant is purified by first removing the cells by centrifugation. The pH is then dropped to about 4 by adding 1 N HCl. The insoluble material is pelleted by centrifugation. Typically, the variant is found in the supernatant. The supernatant is then dialyzed versus 20 mM sodium acetate pH 4. The variant typically precipitates at this step. Both precipitates are resuspended in Tris base and assayed for the inhibition of a Y217L derivative of subtilisin BPN'. In some instances, the variant remains soluble through these precipitation steps. In such a case, the soluble fraction is separated on an S Sepharose column, run in 20 mM sodium acetate pH 4. The sample is eluted with increasing concentrations of sodium chloride and assayed for protease inhibition. In all cases, variant-containing fractions are dialyzed against 1 mM Tris pH 8.0 before use.

Characterization of the Variants of the Present Invention

SSI inhibits, *inter alia*, subtilisin BPN' and a Y217L variant of subtilisin BPN'. Inhibition activity of the present variants is measured as follows, using SSI as an example. SSI is mixed with protease and incubated for fifteen minutes at room temperature in the presence of 0.1 M Tris, pH 8.6, 10 mM $CaCl_2$. Protease activity is then measured using the method of DelMar et al., Analytical Biochemistry, Vol. 99, pp. 316-320 (1979). Addition of 10 μL of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (20 mg/mL) begins the reaction. The reaction rate is measured by the increase in absorbance at 410 nm which indicates inhibition of the protease. The inhibitory properties of the variants of the present invention are similarly determined.

Because it is desirable to incorporate a variant of the present invention with a protease into cleaning or personal care compositions (suitable proteases are described herein infra), the stability in the product environment is also tested. The stability of a variant may be monitored by measuring protease activity over time. If the variant is stable, the level of protease activity will be constant. However, if a variant is destroyed, the protease activity will rise. In this example, variants are mixed with 1.1 nmol of a subtilisin BPN' variant having a Y217L substitution. Water is added so that the volumes of all samples are the same. A complex is allowed to form over ten minutes in a liquid detergent composition made according to the following formula:

Component	Weight Percent
C_{14-15} alkyl (ethoxy 2.25) sulfonic acid	18.0
C_{12-13} alkyl ethoxylate (9)	2.0
C_{12}-N-methylglucamide	5.0
Citric acid	4.0
Ethanol	3.5
Monoethanolamine	2.0
1,2 Propanediol	7.0
Sodium Formate	0.6
Tetraethylene pentamine ethoxylate (16)	1.18
Soil release Polymer	0.15
Silicone Suds suppresser	0.10
Brightener	0.10
Water, NaOH	Balance to 100%

This composition constitutes one-third of the total sample volume. 15 μL of sample is mixed with 975 μL of 0.1 M Tris HCl, pH 8.6, 0.01 M $CaCl_2$. This dilution is incubated for thirty minutes at room temperature. After incubation, substrate is added, and the amount of protease is measured. Degradation of the variant is detected by increase in protease activity over several weeks. Such degradation may be directly compared to that of, for example, SSI.

The K_i of a variant is determined as follows. The variant and 600 μg/mL succinyl-Ala-Ala-Pro-Phe-p-nitroanilide are mixed in 990μL of a 50 mM Tris pH 8 solution. The reaction is started by the addition of a selected protease (suitable proteases are described herein *infra*). The hydrolysis rate is followed over twenty minutes. A constant rate is observed over the last ten to fifteen minutes. This rate, compared to the rate in the absence of variant, is used to calculate the K_i according to the equations of Goldstein, "The Mechanism of Enzyme-Inhibitor-Substrate Reactions", Journal of General Physiology, Vol. 27, pp. 529 - 580 (1944).

Cleaning Compositions of the Present Invention

In another embodiment of the present invention, an effective amount of one or more of the present variants is included in cleaning compositions useful for cleaning a variety of surfaces in need of peptide stain removal. Such cleaning compositions include, but are not limited to, fabric cleaning compositions, hard surface cleansing compositions, light duty cleaning compositions including dish cleansing compositions, and automatic dishwasher detergent compositions.

The cleaning compositions herein comprise an effective amount of one or more variants of the present invention and a cleaning composition carrier, which carrier includes a protease. As used herein, "effective amount of variant", or the like, refers to the quantity of variant necessary to achieve the proteolytic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the particular variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (*e.g.*, granular, bar) composition is desired, and the like. Preferably, the cleaning compositions comprise from about 0.0001% to about 10%, more preferably from about 0.001% to about 1%, and most preferably from about 0.01% to about 0.1% of one or more variants of the present invention. Several examples of various cleaning compositions wherein the variants may be employed are discussed in further detail below.

The present variants are useful in cleaning compositions to inhibit a protease therein during storage, thereby protecting the protease from autolysis or hydrolysis from

other sources such as additional enzymes present in the composition. Accordingly, an essential ingredient in the present cleaning compositions is a protease of which the present variants inhibit. The protease may be of animal, plant or, preferably, microorganism origin. Preferred proteases include those for which SSI is an inhibitor. Such proteases include, for example, those produced by *Bacillus alcalophilus*, *Bacillus amyloliquefaciens*, *Bacillus amylosaccharicus*, *Bacillus licheniformis*, *Bacillus lentus*, and *Bacillus subtilis* microorganisms. Among such proteases the preferred include, for example, subtilisin BPN, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 309, proteinase K, and thermitase, including A/S Alcalase® (Novo Industries, Copenhagen, Denmark), Esperase® (Novo Industries), Savinase® (Novo Industries), Maxatase® (Gist-Brocades, Delft, Netherlands), Maxacal® (Gist-Brocades), Maxapem 15® (Gist-Brocades), and variants of the foregoing. Especially preferred proteases for use herein include those obtained from *Bacillus amyloliquefaciens* and variants thereof. The most preferred wild-type protease is subtilisin BPN'.

Variants of subtilisin BPN', hereinafter collectively referred to as "Protease Group A", are useful as the proteases herein and are disclosed in U.S. Patent No. 5,030,378, Venegas, July 9, 1991 as characterized by the subtilisin BPN' amino acid sequence (the sequence of which is represented as SEQ ID: NO 3) with the following mutations:

(a) Gly at position 166 is substituted with Asn, Ser, Lys, Arg, His, Gln, Ala or Glu; Gly at position 169 is substituted with Ser; and Met at position 222 is substituted with Gln, Phe, His, Asn, Glu, Ala or Thr; or

(b) Gly at position 160 is substituted with Ala, and Met at position 222 is substituted with Ala.

Additional variants of subtilisin BPN', hereinafter collectively referred to as "Protease Group B", are useful as the proteases herein and are disclosed in European Patent EP-B-251,446, assigned to Genencor International, Inc., published January 7, 1988, and granted December 28, 1994, as characterized by the wild-type BPN' amino acid sequence with mutations at one or more of the following positions: Tyr21, Thr22, Ser24, Asp36, Ala45, Ala48, Ser49, Met50, His67, Ser87, Lys94, Val95, Gly97, Ser101,

Gly102, Gly103, Ile107, Gly110, Met 124, Gly127, Gly128, Pro129, Leu135, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, Tyr214, Gly215, and Ser221; or two or more of the positions listed above combined with Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217, and Met222.

Another preferred subtilisin BPN' variant useful as the proteases herein are hereinafter collectively referred to as "Protease Group C", and are described in WO 95/10615, assigned to Genencor International Inc., published April 20, 1995 as characterized by the wild-type subtilisin BPN' amino acid sequence with a mutation to position Asn76, in combination with mutations in one or more other positions selected from the group consisting of Asp99, Ser101, Gln103, Tyr104, Ser105, Ile107, Asn109, Asn123, Leu126, Gly127, Gly128, Leu135, Glu156, Gly166, Glu195, Asp197, Ser204, Gln206, Pro210, Ala216, Tyr217, Asn218, Met222, Ser260, Lys265, and Ala274.

Other preferred subtilisin BPN' variants useful as the proteases herein, collectively referred to as "Protease Group D", are described in U.S. Patent No. 4,760,025, Estell, et al., July 26, 1988, as characterized by the wild-type subtilisin BPN' amino acid sequence with mutations to one or more amino acid positions selected from the group consisting of Asp32, Ser33, His64, Tyr104, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217, and Met222.

The more preferred proteases as used herein are selected from the group consisting of Alcalase®, subtilsin BPN', Protease Group A, Protease Group B, Protease Group C, and Protease Group D. The most preferred protease is selected from Protease Group D.

The present compositions comprise from about 0.0001% to about 1%, preferably from about 0.0005% to about 0.2%, most preferably from about 0.002% to about 0.1%, by weight of the composition, of active protease. Mixtures of protease may also be included. Of course, the weight percent of protease in the cleaning composition will vary depending on the water content, builder content, and the like of the finished composition. For example, it is preferred that in a granular detergent, from about 0.064 mg / g to about 0.64 mg / g of protease in the composition is desirable.

In a preferred embodiment, the preferred molar ratio of variant to protease (variant to protease ratio) in cleaning compositions is from about 3:1 to about 1:1, more preferably from about 3:1 to about 1.5:1, and most preferably about 2:1.

In addition to the present variants, the present cleaning compositions further comprise a cleaning composition carrier comprising one or more cleaning composition materials compatible with the variant and / or the protease. The term "cleaning composition material", as used herein, means any material selected for the particular type of cleaning composition desired and the form of the product (*e.g.*, liquid, granule, bar, spray, stick, paste, gel), which materials are also compatible with the variant used in the composition. The specific selection of cleaning composition materials is readily made by considering the material to be cleaned, the desired form of the composition for the cleaning condition during use. The term "compatible", as used herein, means the cleaning composition materials do not reduce the inhibitory activity of the variant and / or the proteolytic activity of the protease to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

The variants of the present invention may be used in a variety of detergent compositions where high sudsing and good cleansing activity is desired. Thus, the variants can be used with various conventional ingredients to provide fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering compositions, and the like. Such compositions can be in the form of liquids, granules, bars, and the like. Such compositions can be formulated as "concentrated" detergents which contain as much as from about 30% to about 60% by weight of surfactants.

The cleaning compositions herein may optionally, and preferably, contain various surfactants (*e.g.*, anionic, nonionic, or zwitterionic surfactants). Such surfactants are typically present at levels of from about 5% to about 35% of the compositions.

Nonlimiting examples of surfactants useful herein include the conventional C_{11}-C_{18} alkyl benzene sulfonates and primary and random alkyl sulfates, the C_{10}-C_{18} secondary (2,3) alkyl sulfates of the formulas $CH_3(CH_2)_x(CHOSO_3)^-M^+)CH_3$ and $CH_3(CH_2)_y(CHOSO_3^-M^+)\ CH_2CH_3$ wherein x and (y+1) are integers of at least about 7,

preferably at least about 9, and M is a water-solubilizing cation, especially sodium, the C_{10}-C_{18} alkyl alkoxy sulfates (especially EO 1-5 ethoxy sulfates), C_{10}-C_{18} alkyl alkoxy carboxylates (especially the EO 1-5 ethoxycarboxylates), the C_{10}-C_{18} alkyl polyglycosides, and their corresponding sulfated polyglycosides, C_{12}-C_{18} α-sulfonated fatty acid esters, C_{12}-C_{18} alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C_{12}-C_{18} betaines and sulfobetaines ("sultaines"), C_{10}-C_{18} amine oxides, and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. The use of such surfactants in combination with the amine oxide and / or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator. Other conventional useful surfactants are listed in standard texts. Particularly useful surfactants include the C_{10}-C_{18} N-methyl glucamides disclosed in U.S. Patent No. 5, 194,639, Connor et al., issued March 16, 1993.

A wide variety of other ingredients useful in the present cleaning compositions include, for example, other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, and solvents for liquid formulations. If an additional increment of sudsing is desired, suds boosters such as the C_{10}-C_{16} alkolamides can be incorporated into the compositions, typically at about 1% to about 10% levels. The C_{10}-C_{14} monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as $MgCl_2$, $MgSO_4$, and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additional sudsing.

The liquid detergent compositions herein may contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols exemplified by methanol, ethanol, propanol, and *iso*-propanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6 hydroxy groups (*e.g.*, 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions

may contain from about 5% to about 90%, typically from about 10% to about 50% of such carriers.

The detergent compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11. Finished products are typically formulated at this range. Techniques for controlling pH at recommended usage levels include the use of, for example, buffers, alkalis, and acids. Such techniques are well known to those skilled in the art.

When formulating the hard surface cleaning compositions and fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

Likewise, the formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases and proteases in such compositions, typically at levels of from about 0.001% to about 1% by weight. Various detersive and fabric care enzymes are well-known in the laundry detergent art.

Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

Soil release agents, especially of the anionic oligoester type, chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, clay soil removal agents, especially ethoxylated tetraethylene pentamine, dispersing agents, especially polyacrylates and polyasparatates, brighteners, especially anionic brighteners, suds suppressors, especially silicones and secondary alcohols, fabric softeners, especially smectite clays, and the like can all be used in such compositions at levels ranging from about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

Enzyme stabilizers may also be used in the cleaning compositions. Such enzyme stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (preferably from about 0.1% to about 1%).

Other useful cleaning composition materials include clay soil removal agents, dispersing agents, brighteners, suds suppressors, and fabric softeners.

The present variants are useful in hard surface cleaning compositions. As used herein "hard surface cleaning composition" refers to liquid and granular detergent compositions for cleaning hard surfaces such as floors, walls, bathroom tile, and the like. Hard surface cleaning compositions typically comprise a surfactant and a water-soluble sequestering builder. In certain specialized products such as spray window cleaners, however, the surfactants are sometimes not used since they may produce a filmy and / or streaky residue on the glass surface.

The surfactant component, when present, may comprise as little as 0.1% of the compositions herein, but typically the compositions will contain from about 0.25% to about 10%, more preferably from about 1% to about 5% of surfactant.

Typically the compositions will contain from about 0.5% to about 50% of a detergency builder, preferably from about 1% to about 10%.

Preferably the pH should be in the range of from about 7 to about 12. Conventional pH adjustment agents such as sodium hydroxide, sodium carbonate or hydrochloric acid can be used if adjustment is necessary.

Solvents may be included in the compositions. Useful solvents include, but are not limited to, glycol ethers such as diethyleneglycol monohexyl ether, diethyleneglycol monobutyl ether, ethyleneglycol monobutyl ether, ethyleneglycol monohexyl ether, propyleneglycol monobutyl ether, dipropyleneglycol monobutyl ether, and diols such as 2,2,4-trimethyl-1,3-pentanediol and 2-ethyl-1,3-hexanediol. When used, such solvents are typically present at levels of from about 0.5% to about 15%, more preferably from about 3% to about 11%.

Additionally, highly volatile solvents such as *iso*-propanol or ethanol can be used in the present compositions to facilitate faster evaporation of the composition from

surfaces when the surface is not rinsed after "full strength" application of the composition to the surface. When used, volatile solvents are typically present at levels of from about 2% to about 12% in the compositions.

The present variants are also useful for inclusion in the cleaning compositions described in the following: Provisional U.S. Patent Application Serial No. 60/079,477, Rubingh et al., filed March 26, 1998; Provisional U.S. Patent Application Serial No. 60/079,397, Rubingh et al., filed March 26, 1998; U.S. Patent Application Serial No. 09/048,174, Weisgerber et al., filed March 26, 1998; and U.S. Patent Application Serial No. 09/088912, claiming priority to U.S. Patent Application Serial No. 09/048,174, Weisgerber et al., filed June 2, 1998.

Hard surface cleaning compositions of the present invention are illustrated by the following examples.

Examples 1 - 6

Liquid Hard Surface Cleaning Compositions

	Ex. 1	Ex. 2	Ex. 3	Ex. 4	Ex. 5	Ex. 6
Variant 6 - I / Protease	0.05 %	0.50 %	0.02 %	0.03 %	0.30 %	0.05 %
EDTA	-	-	2.90 %	2.90 %	-	-
Sodium Citrate	-	-	-	-	2.90 %	2.90 %
NaC_{12} Alkyl-benzene sulfonate	1.95 %	-	1.95 %	-	1.95 %	-
NaC_{12} Alkylsulfate	-	2.20 %	-	2.20 %	-	2.20 %
NaC_{12} (ethoxy) sulfate	-	2.20 %	-	2.20 %	-	2.20 %
C_{12} Dimethylamine oxide	-	0.50 %	-	0.50 %	-	0.50 %
Sodium cumene sulfonate	1.30 %	-	1.30 %	-	1.30 %	-
Hexyl Carbitol	6.30 %	6.30 %	6.30 %	6.30 %	6.30 %	6.30 %
Water	90.4 %	88.3 %	87.53 %	85.87 %	87.25 %	85.85 %

All formulas are adjusted to pH 7.

In Examples 1 - 6, the variants recited in Tables 7, 8, and 10, and the preferred variants cited herein, among others, are substituted for Variant 6 - I, with substantially similar results.

In another embodiment of the present invention, dishwashing compositions comprise one or more variants of the present invention. As used herein, "dishwashing composition" refers to all forms of compositions for cleaning dishes including, but not limited to, granular and liquid forms. Dishwashing compositions of the present invention are illustrated by the following examples.

Examples 7 - 10

Liquid Dish Detergent

	Ex. 7	Ex. 8	Ex. 9	Ex. 10
Variant 7 - I - A62K / Protease	0.05 %	0.50 %	0.02 %	0.40 %
C_{12} - C_{14} N-methyl glucamide	0.90 %	0.90 %	0.90 %	0.90 %
C_{12} ethoxy (1) sulfate	12.0 %	12.0 %	12.0 %	12.0 %
2-Methyl undecanoic acid	4.50 %	4.50 %	4.50 %	4.50 %
C_{12} ethoxy (2) carboxylate	4.50 %	4.50 %	4.50 %	4.50 %
C_{12} alcohol ethoxylate (4)	3.00 %	3.00 %	3.00 %	3.00 %
C_{12} amine oxide	3.00 %	3.00 %	3.00 %	3.00 %
Sodium cumene sulfonate	2.00 %	2.00 %	2.00 %	2.00 %
Ethanol	4.00 %	4.00 %	4.00 %	4.00 %
Mg^{2+} (as $MgCl_2$)	0.20 %	0.20 %	0.20 %	0.20 %
Ca^{2+} (as $CaCl_2$)	0.40 %	0.40 %	0.40 %	0.40 %
Water	65.45 %	65 %	65.48 %	65.1 %

All formulas are adjusted to pH 7.

In Examples 7 - 10, the variants recited in Tables 7, 8, and 10, and the preferred variants cited herein, among others, are substituted for Variant 7 - I - A62K, with substantially similar results.

Liquid fabric cleaning compositions of the present invention are illustrated by the following examples.

Examples 11 - 13

Liquid Fabric Cleaning Compositions

	Ex. 11	Ex. 12	Ex. 13
Variant 2 - I / Protease	0.05 %	0.03 %	0.30 %
Sodiuam C_{12} - C_{14} alkyl sulfate	20.0 %	20.0 %	20.0 %
2-Butyl octanoic acid	5.0 %	5.0 %	5.0 %
Sodium citrate	1.0 %	1.0 %	1.0 %
C_{10} alcohol ethoxylate (3)	13.0 %	13.0 %	13.0 %
Monoethanolamine	2.50 %	2.50 %	2.50 %
Water/propylene glycol/ethanol (100:1:1)	58.45 %	58.47 %	58.20 %

In Examples 11 - 13, the variants recited in Tables 7, 8, and 10, and the preferred variants cited herein, among others, are substituted for Variant 2 - I, with substantially similar results.

Personal Care Compositions

The present variants are also suited for use in personal care compositions selected from, for example, leave-on and rinse-off hair conditioners, shampoos, leave-on and rinse-off acne compositions, facial milks and conditioners, shower gels, soaps, foaming and non-foaming facial cleansers, cosmetics, hand, facial, and body lotions and moisturizers, leave-on facial moisturizers, cosmetic and cleansing wipes, oral care compositions, and contact lens care compositions. The present personal care compositions comprise and effective amount of one or more variants of the present invention and a personal care carrier, which personal care carrier includes a protease. Effective amounts of variants, including preferred limitations, are described herein with respect to cleaning compositions. Suitable proteases, including those which are preferable, are described herein with respect to cleaning compositions.

To illustrate, the present variants are suitable for inclusion, along with a protease, in the compositions described in the following references: U.S. Pat. No. 5,641,479, Linares et al., issued June 24, 1997 (skin cleansers); U.S. Pat. No. 5,599,549, Wivell et al., issued February 4, 1997 (skin cleansers); U.S. Pat. No. 5,585,104, Ha et al., issued December 17, 1996 (skin cleansers); U.S. Pat. No. 5,540,852, Kefauver et al., issued July

30, 1996 (skin cleansers); U.S. Pat. No. 5,510,050, Dunbar et al., issued April 23, 1996 (skin cleansers); U.S. Pat. No. 5,612,324, Guang Lin et al., issued March 18, 1997 (anti-acne preparations); U.S. Pat. No. 5,587,176, Warren et al., issued December 24, 1996 (anti-acne preparations); U.S. Pat. No. 5,549,888, Venkateswaran, issued August 27, 1996 (anti-acne preparations); U.S. Pat. No. 5,470,884, Corless et al., issued November 28, 1995 (anti-acne preparations); U.S. Pat. No. 5,650,384, Gordon et al., issued July 22, 1997 (shower gels); U.S. Pat. No. 5,607,678, Moore et al., issued March 4, 1997 (shower gels); U.S. Pat. No. 5,624,666, Coffindaffer et al., issued April 29, 1997 (hair conditioners and / or shampoos); U.S. Pat. No. 5,618,524, Bolich et al., issued April 8, 1997 (hair conditioners and / or shampoos); U.S. Pat. No. 5,612,301, Inman, issued March 18, 1997 (hair conditioners and / or shampoos); U.S. Pat. No. 5,573,709, Wells, issued November 12, 1996 (hair conditioners and / or shampoos); U.S. Pat. No. 5,482,703, Pings, issued January 9, 1996 (hair conditioners and / or shampoos); U.S. Pat. No. Re. 34,584, Grote et al., Reissued April 12, 1994 (hair conditioners and / or shampoos); U.S. Pat. No. 5,641,493, Date et al., issued June 24, 1997 (cosmetics); U.S. Pat. No. 5,605,894, Blank et al., issued February 25, 1997 (cosmetics); U.S. Pat. No. 5,585,090, Yoshioka et al., issued December 17, 1996 (cosmetics); U.S. Pat. No. 4,939,179, Cheney et al., issued July 3, 1990 (hand, face, and / or body lotions); U.S. Pat. No. 5,607,980, McAtee et al., issued March 4, 1997 (hand, face, and / or body lotions); U.S. Pat. No. 4,045,364, Richter et al., issued August 30, 1977 (cosmetic and cleansing wipes); European Patent Application, EP 0 619 074, Touchet et al., published October 12, 1994 (cosmetic and cleansing wipes); U.S. Pat. No. 4,975,217, Brown-Skrobot et al., issued December 4, 1990 (cosmetic and cleansing wipes); U.S. Pat. No. 5,096,700, Seibel, issued March 17, 1992 (oral cleaning compositions); U.S. Pat. No. 5,028,414, Sampathkumar, issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 5,028,415, Benedict et al., issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 5,028,415, Benedict et al., issued July 2, 1991 (oral cleaning compositions); U.S. Pat. No. 4,863,627, Davies et al., September 5, 1989 (contact lens cleaning solutions); U.S. Pat. No. Re. 32,672, Huth et al, reissued May 24, 1988 (contact lens cleaning solutions); and U.S. Pat. No. 4,609,493, Schafer, issued September 2, 1986 (contact lens cleaning solutions).

The present variants are also useful for inclusion in the personal care compositions described in the following: Provisional U.S. Patent Application Serial No. 60/079,477, Rubingh et al., filed March 26, 1998; Provisional U.S. Patent Application Serial No. 60/079,397, Rubingh et al., filed March 26, 1998; U.S. Patent Application Serial No. 09/048,174, Weisgerber et al., filed March 26, 1998; and U.S. Patent Application Serial No. 09/088912, claiming priority to U.S. Patent Application Serial No. 09/048,174, Weisgerber et al., filed June 2, 1998.

In a preferred embodiment, the preferred molar ratio of variant to protease (variant to protease ratio) in personal care compositions is from about 3:1 to about 1:1, more preferably from about 3:1 to about 1.5:1, and most preferably about 2:1.

To further illustrate oral cleaning compositions of the present invention, one or more variants of the present invention and one or more proteases are included in compositions useful for removing proteinaceous stains from teeth or dentures. As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment solutions, and the like. Preferably, the oral cleaning compositions comprise from about 0.0001% to about 20% of one or more variants of the present invention, together with a protease, more preferably from about 0.001% to about 10%, more preferably still from about 0.01% to about 5%, by weight of the composition, and a personal care carrier.

Typically, the personal care carrier components of the oral cleaning components of the oral cleaning compositions will generally comprise from about 50% to about 99.99%, preferably from about 65% to about 99.99%, more preferably from about 65% to about 99%, by weight of the composition.

The personal care carrier components and optional components which may be included in the oral cleaning compositions of the present invention are well known to those skilled in the art. A wide variety of composition types, carrier components and optional components useful in the oral cleaning compositions are disclosed in the references cited hereinabove.

In another embodiment of the present invention, denture cleaning compositions for cleaning dentures outside of the oral cavity comprise one or more variants of the

present invention. Such denture cleaning compositions comprise one or more of the variants of the present invention together with a protease, preferably from about 0.0001% to about 50%, more preferably from about 0.001% to about 35%, more preferably still from about 0.01% to about 20%, by weight of the composition, and a personal care carrier. Various denture cleansing composition formats such as effervescent tablets and the like are well known in the art (see, e.g., U.S. Pat. No. 5,055,305, Young), and are generally appropriate for incorporation of one or more of the variants for removing proteinaceous stains from dentures.

In another embodiment of the present invention, contact lens cleaning compositions comprise one or more variants of the present invention. Such contact lens cleaning compositions comprise one or more of the variants, preferably from about 0.01% to about 50% of one or more of the variants, more preferably from about 0.01% to about 20%, more preferably still from about 1% to about 5%, by weight of the composition, and a personal care carrier. Various contact lens cleaning composition formats such as tablets, liquids and the like are well known in the art and are generally appropriate for incorporation of one or more variants of the present invention for removing proteinaceous stains from contact lenses.

The contact lens cleaning composition embodiment of the present invention is illustrated by Examples 14 - 17.

Examples 14 - 17

Contact Lens Cleaning Solution

	Ex. 14	Ex. 15	Ex. 16	Ex. 17
Variant 9 - I / Protease	0.01 %	0.5 %	0.1 %	2.0 %
Glucose	50.0 %	50.0 %	50.0 %	50.0 %
Nonionic surfactant (polyoxyethlene - polyoxypropylene copolymer)	2.0 %	2.0 %	2.0 %	2.0 %
Anionic surfactant (polyoxyethylene - alkylphenylether sodium sulfricester)	1.0 %	1.0 %	1.0 %	1.0 %
Sodium Chloride	1.0 %	1.0 %	1.0 %	1.0 %
Borax	0.30 %	0.30 %	0.30 %	0.30 %
Water	45.69 %	45.20 %	45.60 %	43.70 %

In Examples 14 - 17, the variants recited in Tables 7, 8, and 10, and the preferred variants cited herein, among others, are substituted for Variant 9 - I with substantially similar results.

Examples 18 - 21 illustrate the use of the present variants in bodywash products:

Examples 18 - 21

Bodywash Products

	Ex. 18	Ex. 19	Ex. 20	Ex. 21
Water	62.62 %	65.72 %	57.72 %	60.72 %
Disodium EDTA	0.2 %	0.2 %	0.2 %	0.2 %
Glycerine	3.0 %	3.0 %	3.0 %	3.0 %
Polyquaternium 10	0.4 %	0.4 %	0.4 %	0.4 %
Sodium laureth sulphate	12.0 %	12.0 %	12.0 %	12.0 %
Cocamide MEA	2.8 %	2.8 %	2.8 %	2.8 %
Sodium lauraphoacetate	6.0 %	6.0 %	6.0 %	6.0 %
Myristic Acid	1.6 %	1.6 %	1.6 %	1.6 %
Magnesium sulphate heptahydrate	0.3 %	0.3 %	0.3 %	0.3 %

Trihydroxystearin	0.5 %	0.5 %	0.5 %	0.5 %
PEG-6 caprylic / capric triglycerides	3.0 %	-	-	-
Sucrose polyesters of cottonate fatty acid	3.0 %	-	-	-
Sucrose polyesters of behenate fatty acid	3.0 %	-	4.0 %	-
Petrolatum	-	4.0 %	8.0 %	-
Mineral Oil	-	-	-	6.0 %
DMDM Hydantoin	0.08 %	0.08 %	0.08 %	0.08 %
Variant 14 - I / Protease	0.1 %	2.0 %	2.0 %	5.0 %
Citric Acid	1.40 %	1.40 %	1.40 %	1.40 %

In Examples 18 - 21, the variants recited in Tables 7, 8, and 10, and the preferred variants cited herein, among others, are substituted for Variant 14 - I, with substantially similar results.

Examples 18 - 21 illustrate the use of the present variants in facewash products:

EXAMPLES 22 - 25

Facewash Products

	Ex. 22	Ex. 23	Ex. 24	Ex. 25
Water	66.52 %	65.17 %	68.47 %	68.72 %
Disodium EDTA	0.1 %	0.1 %	0.2 %	0.2 %
Citric Acid	-	-	1.4 %	1.4 %
Sodium Laureth-3 Sulfate	3.0 %	3.5 %	-	-
Sodium Laureth-4 Carboxylate	3.0 %	3.5 %	-	-
Laureth-12	1.0 %	1.2 %	-	-
Polyquaternium 10	-	-	0.4 %	0.4 %
Polyquaternium 25	0.3 %	0.3 %	-	-
Glycerine	3.0 %	3.0 %	3.0 %	3.0 %

Sodium Lauroamphoacetate	-	-	6.0 %	6.0 %
Lauric Acid	6.0 %	6.0 %	3.0 %	3.0 %
Myristic Acid	-	-	3.0 %	3.0 %
Magnesium sulphate heptahydrate	2.3 %	2.0 %	2.0 %	2.0 %
Triethanol amine	4.0 %	4.0 %	4.0 %	4.0 %
Trihydroxystearin	0.5 %	0.5 %	0.5 %	0.5 %
Sucrose polyesters of behenate fatty acid	2.0 %	2.0 %	-	-
Sucrose polyesters of cottonate fatty acid	3.0 %	2.0 %	-	-
PEG-6 caprylic / capric triglycerides	-	-	-	2.0 %
Petrolatum	-	-	4.0 %	-
Mineral Oil	-	-	-	2.0 %
Cocamidopropyl betaine	2.0 %	3.0 %	1.8 %	1.8 %
Lauryl dimethylamine oxide	1.0 %	1.2 %	1.2 %	1.2 %
Dex Panthenol	1.0 %	0.25 %	0.25 %	-
DMDM Hydantoin	0.08 %	0.08 %	0.08 %	0.08 %
Variant 24 - I / Protease	1.0 %	2.0 %	0.5 %	0.5 %
Fragrance	0.2 %	0.2 %	0.2 %	0.2 %

In Examples 22 - 25, the variants recited in Tables 7, 8, and 10, and the preferred variants cited herein, among others, are substituted for Variant 24 - I, with substantially similar results.

Examples 26 - 27 illustrate the use of the present variants in leave-on skin moisturizing compositions:

EXAMPLES 26 - 27

Leave-on Skin Moisturizing Composition

	Ex. 26	Ex. 27
Glycerine	5.0 %	-
Stearic acid	3.0 %	-
C_{11-13} Isoparaffin	2.0 %	-
Glycol stearate	1.5 %	-
Propylene glycol	-	3.0 %
Mineral oil	1.0 %	10.0 %
Sesame oil	-	7.0 %
Petrolatum	-	1.8 %
Triethanolamine	0.7 %	-
Cetyl acetate	0.65 %	-
Glyceryl stearate	0.48 %	2.0 %
TEA stearate	-	2.5 %
Cetyl alcohol	0.47 %	-
Lanolin alcohol	-	1.8 %
DEA - cetyl phosphate	0.25 %	-
Methylparaben	0.2 %	0.2 %
Propylparaben	0.12 %	0.1 %
Carbomer 934	0.11 %	-
Disodium EDTA	0.1 %	-
Variant 13 - I / Protease	0.1 %	0.5 %
Water	84.32 %	71.1 %

In Examples 26 - 27, the variants recited in Tables 7, 8, and 10, and the preferred variants cited herein, among others, are substituted for Variant 13 - I with substantially similar results.

Example 28 illustrates the use of the present variants in cleansing wipe commpositions:

EXAMPLE 28

Cleansing Wipe Composition

Propylene Glycol	1.0 %
Ammonium lauryl sulfate	0.6 %
Succinic acid	4.0 %
Sodium succinate	3.2 %
Triclosan®	0.15 %
Variant 20 - I / Protease	0.05 %
Water	91.0 %

The above composition is impregnated onto a woven absorbent sheet comprised of cellulose and / or polyester at about 250%, by weight of the absorbent sheet.

In Example 28, the variants recited in Tables 7, 8, and 10, and the preferred variants cited herein, among others, are substituted for Variant 20 - I with substantially similar results.

What is claimed is:

1. A variant characterized by a modified amino acid sequence of a parent amino acid sequence, wherein the modified amino acid sequence is characterized by an amino acid substitution at position 63 corresponding to SSI, and wherein the parent amino acid sequence is selected from the group consisting of SSI, SSI-like inhibitors, variants of SSI, and variants of SSI-like inhibitors.

2. A variant according to Claim 1 wherein the amino acid substitution at position 63 corresponding to SSI is with isoleucine.

3. A variant according to any of the preceding claims wherein the parent amino acid sequence is selected from the group consisting of SSI and variants of SSI.

4. A variant according to any of the preceding claims which exhibits a K_i such that the variant:
   (a) inhibits a protease in a composition comprising the variant and the protease; and
   (b) dissociates from the protease upon dilution.

5. A variant according to any of the preceding claims which exhibits a K_i is from about 10^{-12} M to about 10^{-4} M.

6. A variant according to any of the preceding claims selected from the group consisting of:
   (a) L63I + D83C;
   (b) L63I + M73D;
   (c) L63I + M73D + D83C;
   (d) L63I + M73P + D83C;
   (e) L63I + M70Q + D83C;

(f) L63I + M70Q + M73P + V74F + D83C;

(g) L63I + M70Q + M73P + V74W + D83C;

(h) L63I + M70Q + M73P + D83C + S98A;

(i) L63I + G47D + M73P + V74F + D83C;

(j) L63I + G47D + M73P + V74W + D83C;

(k) L63I + G47D + M73P + D83C + S98A;

(l) L63I + G47D + M70Q + M73P + V74F + D83C;

(m) L63I + G47D + M70Q + M73P + V74W + D83C;

(n) L63I + G47D + M73P + V74F + D83C + S98A;

(o) L63I + G47D + M73P + V74W + D83C + S98A;

(p) A62* + L63I + D83C;

(q) A62* + L63I + M73D;

(r) A62* + L63I + M73D + D83C;

(s) A62* + L63I + M73P + D83C;

(t) A62* + L63I + M70Q + D83C;

(u) A62* + L63I + M73P + D83C + S98A;

(v) A62* + L63I + M73P + Y75A + D83C;

(w) A62* + L63I + M73P + D83C + S98V;

(x) A62* + L63I + M70Q + M73P + D83C;

(y) A62* + L63I + M73P + V74A + D83C;

(z) A62* + L63I + M73P + V74F + D83C;

(aa) A62* + L63I + M70Q + D83C + S98A;

(bb) A62* + L63I + G47D + M70Q + D83C;

(cc) A62* + L63I + G47D + D83C + S98A;

(dd) A62* + L63I + G47D + M73P + D83C;

(ee) A62* + L63I + G47D + M73D + D83C;

(ff) A62* + L63I + M70Q + M73P + V74F + D83C;

(gg) A62* + L63I + M70Q + M73P + V74W + D83C;

(hh) A62* + L63I + M70Q + M73P + D83C + S98A;

(ii) A62* + L63I + G47D + M73P + V74F + D83C;

(jj) A62* + L63I + G47D + M73P + V74W + D83C;

(kk) A62* + L63I + G47D + M73P + D83C + S98A;

(ll) A62* + L63I + G47D + M70Q + M73P + V74F + D83C;

(mm) A62* + L63I + G47D + M70Q + M73P + V74W + D83C;

(nn) A62* + L63I + G47D + M73P + V74F + D83C + S98A;

(oo) A62* + L63I + G47D + M73P + V74W + D83C + S98A;

(pp) L63I + A62K + S98Q;

(qq) L63I + A62K + S98D;

(rr) L63I + A62K + S98E;

(ss) L63I + A62R + S98Q;

(tt) L63I + A62R + S98D;

(uu) L63I + A62R + S98E;

(vv) L63I + S98A;

(ww) L63I + M73P + D83C + S98D;

(xx) L63I + M73P + D83C + S98E;

(yy) L63I + M73P + S98D;

(zz) L63I + M73P + S98E;

(aaa) L63I + M73P + S98A;

(bbb) A62K + L63I + M73P + D83C + S98D;

(ccc) A62R + L63I + M73P + D83C + S98D;

(ddd) A62K + L63I + M73P + D83C + S98E;

(eee) A62R + L63I + M73P + D83C + S98E;

(fff) A62K + L63I + M73P + S98A;

(ggg) A62R + L63I + M73P + S98A;

(hhh) L63I + G47D + M73P + D83C + S98D;

(iii) L63I + G47D + M73P + D83C + S98E; and

(jjj) L63I + M73P.

7. A variant according to any of the preceding claims selected from the group consisting of:

(a) A62K + L63I + M73P + D83C + S98Q;

(b) A62K + L63I + M73P + D83C + S98D;

(c) A62K + L63I + M73P + D83C + S98E;

(d) A62K + L63I + S98Q;

(e) A62K + L63I + S98D;

(f) A62K + L63I + S98E;

(g) A62R + L63I + M73P + D83C + S98Q;

(h) A62R + L63I + M73P + D83C + S98D;

(i) A62R + L63I + M73P + D83C + S98E;

(j) L63I + M73P + D83C + S98A.

(k) A62R + L63I + S98Q;

(l) A62R + L63I + S98D;

(m) A62R + L63I + S98E;

(n) L63I + S98A;

(o) A62K + L63I + M73P + D83C + S98D;

(p) A62R + L63I + M73P + D83C + S98D;

(q) A62K + L63I + M73P + D83C + S98E;

(r) A62R + L63I + M73P + D83C + S98E;

(s) A62K + L63I + M73P + S98A; and

(t) A62R + L63I + M73P + S98A.

8. A mutant gene encoding a variant according to any of the preceding claims.

9. A composition comprising a variant according to any of the preceding claims and a carrier selected from the group consisting of a cleaning composition carrier and a personal care carrier.

10. A composition according to Claim 9 wherein the variant to protease ratio is from about 3:1 to about 1:1.

SEQUENCE LISTING

<110> Saunders, Charles W.
Correa, Paul E.
Sun, Yiping
Rubingh, Donn N.

<120> Stabilized Variants of Streptomyces Subtilisin Inhibitor

<130> Stabilized Variants

<140>
<141>

<150> 60/091,911
<151> 1998-07-07

<160> 15

<170> PatentIn Ver. 2.0

<210> 1
<211> 113
<212> PRT
<213> Streptomyces albogriseolus

<400> 1
Asp Ala Pro Ser Ala Leu Tyr Ala Pro Ser Ala Leu Val Leu Thr Val
1 5 10 15

Gly Lys Gly Val Ser Ala Thr Thr Ala Ala Pro Glu Arg Ala Val Thr
20 25 30

Leu Thr Cys Ala Pro Gly Pro Ser Gly Thr His Pro Ala Ala Gly Ser
35 40 45

Ala Cys Ala Asp Leu Ala Ala Val Gly Gly Asp Leu Asn Ala Leu Thr
50 55 60

Arg Gly Glu Asp Val Met Cys Pro Met Val Tyr Asp Pro Val Leu Leu
65 70 75 80

Thr Val Asp Gly Val Trp Gln Gly Lys Arg Val Ser Tyr Glu Arg Val
85 90 95

Phe Ser Asn Glu Cys Glu Met Asn Ala His Gly Ser Ser Val Ala Phe
100 105 110

Phe

<210> 2
<211> 117
<212> PRT
<213> Streptomyces albogriseolus

<400> 2
Ala Gly Glu Phe Asp Ala Pro Ser Ala Leu Tyr Ala Pro Ser Ala Leu
1 5 10 15

Val Leu Thr Val Gly Lys Gly Val Ser Ala Thr Thr Ala Ala Pro Glu
20 25 30

Arg Ala Val Thr Leu Thr Cys Ala Pro Gly Pro Ser Gly Thr His Pro
35 40 45

```
Ala Ala Gly Ser Ala Cys Ala Asp Leu Ala Ala Val Gly Gly Asp Leu
     50                  55                  60

Asn Ala Leu Thr Arg Gly Glu Asp Val Met Cys Pro Met Val Tyr Asp
 65                  70                  75                  80

Pro Val Leu Leu Thr Val Asp Gly Val Trp Gln Gly Lys Arg Val Ser
                 85                  90                  95

Tyr Glu Arg Val Phe Ser Asn Glu Cys Glu Met Asn Ala His Gly Ser
            100                 105                 110

Ser Val Phe Ala Phe
        115
```

<210> 3
<211> 275
<212> PRT
<213> Bacillus amyloliquefaciens

<400> 3

```
Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu
  1               5                  10                  15

His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
             20                  25                  30

Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
         35                  40                  45

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
     50                  55                  60

Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
 65                  70                  75                  80

Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
                 85                  90                  95

Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
            100                 105                 110

Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
        115                 120                 125

Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
    130                 135                 140

Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
145                 150                 155                 160

Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
                165                 170                 175

Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
            180                 185                 190

Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
        195                 200                 205

Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
    210                 215                 220

Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
225                 230                 235                 240

Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
```

```
                245                 250                 255

Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
            260                 265                 270

Ala Ala Gln
        275


<210> 4
<211> 107
<212> PRT
<213> Streptomyces thermotolerans

<400> 4
Tyr Ala Pro Ser Ala Leu Val Leu Thr Val Gly His Gly Glu Ser Ala
  1               5                  10                  15

Ile Ala Ala Thr Pro Glu Arg Ala Val Thr Leu Thr Cys Ala Pro Lys
             20                  25                  30

Ala Ala Gly Thr His Pro Ala Ala Gly Ala Ala Cys Ala Glu Leu Arg
         35                  40                  45

Gly Val Gly Gly Asp Phe Asp Ala Leu Thr Ala Arg Asp Gly Val Met
     50                  55                  60

Cys Thr Lys Gln Tyr Asp Pro Val Val Val Thr Val Glu Gly Val Trp
 65                  70                  75                  80

Gln Gly Lys Arg Val Ser Tyr Glu Arg Thr Phe Ser Asn Asp Cys Met
                 85                  90                  95

Lys Asn Ala Tyr Gly Thr Gly Val Phe Ser Phe
            100                 105


<210> 5
<211> 109
<212> PRT
<213> Streptomyces galbus

<400> 5
Ser Leu Tyr Ala Pro Ser Ala Leu Val Leu Thr Met Gly His Gly Glu
  1               5                  10                  15

Ser Ala Ala Ala Val Ser Pro Ala Arg Ala Val Thr Leu Asn Cys Ala
             20                  25                  30

Pro Ser Ala Ser Gly Thr His Pro Ala Pro Ala Leu Ala Cys Ala Glu
         35                  40                  45

Leu Arg Ala Ala Gly Gly Asp Leu Asp Ala Leu Ala Gly Pro Ala Asp
     50                  55                  60

Thr Val Cys Thr Lys Gln Tyr Ala Pro Val Val Ile Thr Val Asp Gly
 65                  70                  75                  80

Val Trp Gln Gly Lys Arg Val Ser Tyr Glu Arg Thr Phe Ala Asn Gly
                 85                  90                  95

Cys Val Lys Asn Ala Ser Gly Ser Ser Val Phe Ala Phe
            100                 105


<210> 6
<211> 107
<212> PRT
```

<213> Streptomyces azureus

<400> 6
Tyr Ala Pro Ser Ala Leu Val Leu Thr Val Gly Glu Gly Glu Ser Ala
1 5 10 15

Ala Ala Ala Thr Pro Glu Arg Ala Val Thr Leu Thr Cys Ala Pro Arg
20 25 30

Pro Ser Gly Thr His Pro Val Ala Gly Ser Ala Cys Ala Glu Leu Arg
35 40 45

Gly Val Gly Gly Asp Val His Ala Leu Thr Ala Thr Asp Gly Val Met
50 55 60

Cys Thr Lys Gln Tyr Asp Pro Val Val Val Thr Val Asp Gly Val Trp
65 70 75 80

Gln Gly Arg Arg Val Ser Tyr Glu Arg Thr Phe Ser Asn Glu Cys Val
85 90 95

Lys Asn Ala Tyr Gly Ser Gly Val Phe Ala Phe
100 105

<210> 7
<211> 110
<212> PRT
<213> Streptomyces lividans

<400> 7
Ser Leu Tyr Ala Pro Ser Ala Leu Val Leu Thr Val Gly His Gly Glu
1 5 10 15

Ser Ala Ala Thr Ala Ala Pro Leu Arg Ala Val Thr Leu Thr Cys Ala
20 25 30

Pro Thr Ala Ser Gly Thr His Pro Ala Ala Ala Ala Ala Cys Ala Glu
35 40 45

Leu Arg Ala Ala His Gly Asp Pro Ser Ala Leu Ala Ala Glu Asp Ser
50 55 60

Val Met Cys Thr Arg Glu Tyr Ala Pro Val Val Val Thr Val Asp Gly
65 70 75 80

Val Trp Gln Gly Arg Arg Leu Ser Tyr Glu Arg Thr Phe Ala Asn Glu
85 90 95

Cys Val Lys Asn Ala Gly Ser Ala Ser Val Phe Thr Phe Glu
100 105 110

<210> 8
<211> 110
<212> PRT
<213> Streptomyces longisporus

<400> 8
Ala Ser Leu Tyr Ala Pro Ser Ala Leu Val Leu Thr Val Gly His Gly
1 5 10 15

Thr Ser Ala Ala Ala Ala Thr Pro Leu Arg Ala Val Thr Leu Asn Cys
20 25 30

Ala Pro Thr Ala Ser Gly Thr His Pro Ala Pro Ala Leu Ala Cys Ala
35 40 45

```
Asp Leu Arg Gly Val Gly Gly Asp Ile Asp Ala Leu Lys Ala Arg Asp
     50                  55                  60

Gly Val Ile Cys Asn Lys Leu Tyr Asp Pro Val Val Val Thr Val Asp
 65                  70                  75                  80

Gly Val Trp Gln Gly Lys Arg Val Ser Tyr Glu Arg Thr Phe Gly Asn
                 85                  90                  95

Glu Cys Val Lys Asn Ser Tyr Gly Thr Ser Leu Phe Ala Phe
            100                 105                 110


<210> 9
<211> 113
<212> PRT
<213> Streptomyces parvulus

<400> 9
Thr Ala Pro Ala Ser Leu Tyr Ala Pro Ser Ala Leu Val Leu Thr Ile
  1               5                  10                  15

Gly Gln Gly Glu Ser Ala Ala Ala Thr Ser Pro Leu Arg Ala Val Thr
            20                  25                  30

Leu Thr Cys Ala Pro Lys Ala Thr Gly Thr His Pro Ala Ala Asp Ala
         35                  40                  45

Ala Cys Ala Glu Leu Arg Arg Ala Gly Gly Asp Phe Asp Ala Leu Ser
     50                  55                  60

Ala Ala Asp Gly Val Met Cys Thr Arg Glu Tyr Ala Pro Val Val Val
 65                  70                  75                  80

Thr Val Asp Gly Val Trp Gln Gly Arg Arg Leu Ser Tyr Glu Arg Thr
                 85                  90                  95

Phe Ala Asn Glu Cys Val Lys Asn Ala Gly Ser Ala Ser Val Phe Thr
            100                 105                 110

Phe


<210> 10
<211> 107
<212> PRT
<213> Streptomyces coelicolor

<400> 10
Tyr Ala Pro Ser Ala Leu Val Leu Thr Val Gly His Gly Glu Ser Ala
  1               5                  10                  15

Ala Thr Ala Ala Pro Leu Arg Ala Val Thr Leu Thr Cys Ala Pro Thr
            20                  25                  30

Ala Ser Gly Thr His Pro Ala Ala Asp Ala Ala Cys Ala Glu Leu Arg
         35                  40                  45

Ala Ala His Gly Asp Pro Ser Ala Leu Ala Ala Asp Asp Ala Val Met
     50                  55                  60

Cys Thr Arg Glu Tyr Ala Pro Val Val Val Thr Val Asp Gly Val Trp
 65                  70                  75                  80

Gln Gly Arg Arg Leu Ser Tyr Glu Arg Thr Phe Ala Asn Glu Cys Val
                 85                  90                  95
```

```
Lys Asn Ala Gly Ser Ala Ser Val Phe Thr Phe
            100                 105


<210> 11
<211> 116
<212> PRT
<213> Streptomyces lavendulae

<400> 11
Ala Pro Asp Ala Ala Pro Ala Ser Leu Tyr Ala Pro Ser Ala Leu Val
  1               5                  10                  15

Leu Thr Ile Gly His Gly Gly Ala Ala Ala Thr Ala Thr Pro Glu Arg
             20                  25                  30

Ala Val Thr Leu Thr Cys Ala Pro Thr Ser Ser Gly Thr His Pro Ala
         35                  40                  45

Ala Ser Ala Ala Cys Ala Glu Leu Arg Gly Val Gly Gly Asp Phe Ala
     50                  55                  60

Ala Leu Lys Ala Arg Asp Asp Val Trp Cys Asn Lys Leu Tyr Asp Pro
 65                  70                  75                  80

Val Val Val Thr Ala Gln Gly Val Trp Gln Gly Gln Arg Val Ser Tyr
                 85                  90                  95

Glu Arg Thr Phe Gly Asn Ser Cys Glu Arg Asp Ala Val Gly Gly Ser
            100                 105                 110

Leu Phe Ala Phe
        115


<210> 12
<211> 109
<212> PRT
<213> Streptomyces antifibrinolyticus

<400> 12
Gly Leu Tyr Ala Pro Ser Ala Leu Val Leu Thr Met Gly His Gly Asn
  1               5                  10                  15

Ser Ala Ala Thr Val Asn Pro Glu Arg Ala Val Thr Leu Asn Cys Ala
             20                  25                  30

Pro Thr Ala Ser Gly Thr His Pro Ala Ala Leu Gln Ala Cys Ala Glu
         35                  40                  45

Leu Arg Gly Ala Gly Gly Asp Phe Asp Ala Leu Thr Val Arg Gly Asp
     50                  55                  60

Val Ala Cys Thr Lys Gln Phe Asp Pro Val Val Val Thr Val Asp Gly
 65                  70                  75                  80

Val Trp Gln Gly Lys Arg Val Ser Tyr Glu Arg Thr Phe Ala Asn Glu
                 85                  90                  95

Cys Val Lys Asn Ser Tyr Gly Met Thr Val Phe Thr Phe
            100                 105


<210> 13
<211> 107
<212> PRT
<213> Streptomyces lividans
```

<400> 13
Tyr Ala Pro Ser Ala Leu Val Leu Thr Val Gly His Gly Glu Ser Ala
1 5 10 15

Ala Thr Ala Ala Pro Leu Arg Ala Val Thr Leu Thr Cys Ala Pro Thr
20 25 30

Ala Ser Gly Thr His Pro Ala Ala Ala Ala Ala Cys Ala Glu Leu Arg
35 40 45

Ala Ala His Gly Asp Pro Ser Ala Leu Ala Ala Glu Asp Ser Val Met
50 55 60

Cys Thr Arg Glu Tyr Ala Pro Val Val Val Thr Val Asp Gly Val Trp
65 70 75 80

Gln Gly Arg Arg Leu Ser Tyr Glu Arg Thr Phe Ala Asn Glu Cys Val
85 90 95

Lys Asn Ala Gly Ser Ala Ser Val Phe Thr Phe
100 105

<210> 14
<211> 110
<212> PRT
<213> Streptoverticillium cinnamoneum

<400> 14
Ser Leu Tyr Ala Pro Ser Ala Leu Val Leu Thr Ile Gly Gln Gly Asp
1 5 10 15

Ser Ala Ala Ala Ala Gly Ile Gln Arg Ala Val Thr Leu Thr Cys Met
20 25 30

Pro Lys Ala Asp Gly Thr His Pro Asn Thr Arg Gly Ala Cys Ala Gln
35 40 45

Leu Arg Leu Ala Gly Gly Asp Phe Glu Lys Val Thr Lys Ile Lys Glu
50 55 60

Gly Thr Ala Cys Thr Arg Glu Trp Asn Pro Ser Val Val Thr Ala Glu
65 70 75 80

Gly Val Trp Glu Gly Arg Arg Val Ser Phe Glu Arg Thr Phe Ala Asn
85 90 95

Pro Cys Glu Leu Lys Ala Gly Lys Gly Thr Val Phe Glu Phe
100 105 110

<210> 15
<211> 110
<212> PRT
<213> Streptomyces cacaoi

<400> 15
Ser Leu Tyr Ala Pro Ser Ala Val Val Ile Ser Lys Thr Gln Gly Ala
1 5 10 15

Ser Ala Asp Ala Pro Ala Gln Arg Ala Val Thr Leu Arg Cys Leu Pro
20 25 30

Val Gly Gly Asp His Pro Ala Pro Glu Lys Ala Cys Ala Ala Leu Arg
35 40 45

Glu Ala Gly Gly Asp Pro Ala Ala Leu Pro Arg Tyr Val Glu Asp Thr
50 55 60

```
Gly Arg Val Cys Thr Arg Glu Tyr Arg Pro Val Thr Val Ser Val Gln
 65                  70                  75                  80

Gly Val Trp Asp Gly Arg Arg Ile Asp His Ala Gln Thr Phe Ser Asn
                 85                  90                  95

Ser Cys Glu Leu Glu Lys Gln Thr Ala Ser Val Tyr Ala Phe
            100                 105                 110
```

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :		(11) International Publication Number:	WO 00/01826
C12N 15/31, C07K 14/81, C11D 3/386, 3/33	A3	(43) International Publication Date:	13 January 2000 (13.01.00)

(21) International Application Number: PCT/US99/15246

(22) International Filing Date: 7 July 1999 (07.07.99)

(30) Priority Data:
60/091,911 7 July 1998 (07.07.98) US

(71) Applicant *(for all designated States except US)*: THE PROCTER & GAMBLE COMPANY [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SAUNDERS, Charles, Winston [US/US]; 5561 Carlsbad Court, Fairfield, OH 45014 (US). CORREA, Paul, Elliott [US/US]; 5755 Dry Ridge Road, Cincinnati, OH 45252 (US). SUN, Yiping [US/US]; 7589 Lakota Springs Drive, West Chester, OH 45069 (US). BAUER, Mark, Donald [US/US]; 6832 Richard Avenue, Cincinnati, OH 45224 (US). RUBINGH, Donn, Nelton [US/US]; 8113 Sheed Road, Cincinnati, OH 45247 (US).

(74) Agents: REED, T., David et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217–1087 (US).

(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
6 April 2000 (06.04.00)

(54) **Title:** STABILIZED VARIANTS OF *STREPTOMYCES* SUBTILISIN INHIBITOR

(57) **Abstract**

The present invention relates to variants of *Streptomyces* subtilisin inhibitor (SSI) and those inhibitors having homology to SSI. Such variants are useful in conjunction with enzymes, particularly proteases, in cleaning compositions and personal care compositions. The variants comprise an amino acid substitution at position 63 corresponding to SSI. Such variants provide greater proteolytic stability in cleaning compositions and personal care compositions. The present invention also relates to cleaning compositions and personal care compositions comprising the present variants, as well as genes encoding the variants.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 99/15246

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/81 C11D3/386 C11D3/33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MITSUI Y ET AL: "CRYSTAL STRUCTURE OF A BACTERIAL PROTEIN PROTEINASE INHIBITOR STREPTOMYCES SUBTILISIN INHIBITOR AT 2.6 ANGSTROM RESOLUTION." J MOL BIOL, (1979) 131 (4), 697-724. , XP000867414	1,3,8
Y	page 704, paragraph 3 ---	9,10
Y	WO 98 13387 A (CORREA PAUL ELLIOTT ;LASKOWSKI MICHAEL JR (US); PROCTER & GAMBLE () 2 April 1998 (1998-04-02) cited in the application the whole document ---	9,10

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 2000	04/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

3

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/US 99/15246

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
A	KOJIMA, SHUICHI ET AL: "Effects of deletion in the flexible loop of the protease inhibitor SSI (Streptomyces subtilisin inhibitor) on interactions with proteases." PROTEIN ENGINEERING, (1993) VOL. 6, NO. 3, PP. 297-303. , XP002128269	

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 99/15246

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9813387 A	02-04-1998	EP 0929577 A	21-07-1999